

Placentation and Expression of Homeobox Genes in Extravillous Trophoblasts in First Trimester Pregnancies

Literature review and a morphologic and immunohistochemical pilot study

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**Student thesis at Faculty of Medicine,
University of Oslo, March 2006**

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Table of contents

- 1. Abstract**
- 2. Aim of project work**
- 3. Placentation and homeobox genes**
 - 3.1. Implantation
 - 3.2. Establishing the maternal-fetal circulation barrier
 - 3.3. Different cytotrophoblast cell types
 - 3.4. Remodelling of maternal spiral arteries
 - 3.5. Homeobox genes
 - 3.6. Importance of homeobox genes in placental development
 - 3.7. Homeobox genes in pathological first trimester EVT
- 4. Pilot study**
 - 4.1. Aim of study
 - 4.2. Patient material
 - 4.3. Methods used at Ullevål University Hospital
 - 4.4. Immunohistochemical protocol at Pregnancy Research Centre
 - 4.5. Morphological and immunohistochemical evaluation
- 5. Results**
- 6. Discussion and conclusion**
- 7. References**

1. Abstract

The first part of the thesis comprises a literature review on placental development with focus on trophoblast function and homeobox genes. Homeobox genes belong to a large family of transcription factors that control proliferation, invasion, migration and differentiation. Trophoblast dysfunction is thought to be a major factor in defect placentation and thus important in a number of clinically complicated pregnancies.

The second part of the thesis relates to a morphological and immunohistochemical pilot study on remodelled spiral arteries in the placental bed from first trimester pregnancies. Remodelled spiral arteries were identified morphologically in archive curettage tissue material from Ullevål University Hospital in normal and pathological first trimester pregnancies (therapeutic abortions and spontaneous or missed abortions). The remodelled spiral arteries were identified morphologically in routine stained sections and immunohistochemically by means of protein markers to trophoblasts (CK7) and smooth muscle cells (actin). In an immunohistochemical pilot study at Pregnancy Research Centre at the University of Melbourne we tested the quality of two laboratory made antibodies against the proteins coded for by homeobox genes HLX1 and DLX4 on formalin fixed, paraffin embedded archive tissue. The aim was to explore the protein expression of these two important homeobox genes on the archive material from the normal and pathological first trimester pregnancies. Unfortunately, the antibodies were not found suitable for this use. The problems and pitfalls in protein expression studies on tissue material (immunohistochemistry) are discussed.

2. Aim of project work

Placenta is a transient organ of fundamental importance for the growing fetus. The key cells for the formation and the function of placenta are the trophoblast cells from the developing conceptus.

The aim of this project work was to review the literature on important aspects of placentation with focus on trophoblast action and homeobox genes.

In a pilot study we additionally wanted learn how to identify remodelled spiral arteries in tissue sections from first trimester placental beds. We wanted to test the potentials of laboratory made (PRC, Melbourne) antibodies against the gene products of HLX1 and DLX4. Finally, we wanted to see if any differences could be recorded between the immunoreactivity in extravillous trophoblasts (EVT) in normal and pathological first trimester pregnancies.

3. Background

The placenta connects the mother and the fetus. It has functions related to nutrient and gas exchange, excretion and hormone production. Additionally, it acts as an immunological barrier between the two genetically different individuals. The placenta develops from the chorionic sac, in close proximity to the maternal placental bed, consisting of decidually transformed endometrial stroma with invading, intermediate (extravillous) trophoblasts. In order to understand the development of the placenta, it is necessary to describe the major biological events of reproduction. (Moore and Persaud 1998).

3.1 Implantation

The fertilized egg starts dividing while it travels through the fallopian tube. When it consists of 12-16 cells it is referred to as the morula. Already at this stage the outer cells of the morula are determined to form the placental. The 4th day after fertilisation the structure is called a blastocyst, and the cells show obvious signs of diverse differentiation, consisting of an inner cell mass (embryoblasts) and an outer sphere, with primitive trophectoderm. The inner cell mass cells will later form the embryo, while the primitive trophectoderm will form the placenta.

At day 5 ½ to 6 following fertilisation, the outer layer of trophoblasts start expressing adhesion molecules and the blastocyst adhere to the nearby surface, normally the endometrium in the upper posterior wall of the uterine cavity (Fig. 1A). The outer cells of the adherent trophoblast proliferate and differentiate into the two important trophoblast cell types; the inner cytotrophoblast layer and the outer syncytiotrophoblast layer (Fig. 1B) and the syncytiotrophoblasts start invading the endometrium, superficially implanting the embryo. (Moore and Persaud 1998). The site of implantation is important for the clinical outcome. If the invasion is in the lower part of the uterus, the result is placenta praevia. If it is in the fallopian tube, the result is an ectopic pregnancy.

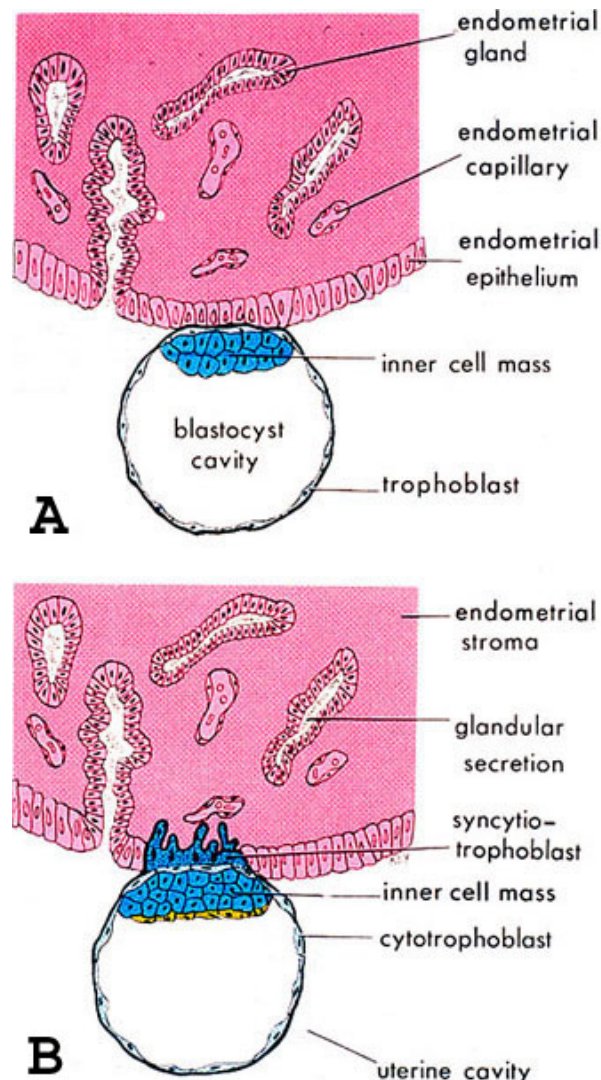


Fig. 1A and B. Blastocyst events at day 5 and 6 following fertilisation.

From Moore KL: *The developing human*. Fourth Edition. W.B. Saunders Company; 1988 p.34.

3.2 Establishing the maternal-fetal circulation barrier

As invasion continues, isolated spaces called lacunae form in the syncytium. Due to trophoblast infiltration into maternal capillaries, maternal blood will fill the lacunae, establishing a primitive exchange between mother and embryo. Proliferating cytotrophoblasts now begin to migrate into the syncytium, some differentiating into syncytiotrophoblasts. This arrangement, with an outer layer of syncytiotrophoblasts and an inner layer of cytotrophoblasts, is referred to as primary villus formation (Fig. 2A). Later, these protrusions are filled with chorionic mesoderm forming the secondary villi (Fig. 2B). Three weeks after fertilisation, the villi are vascularized by fetal capillaries, and are now called tertiary villi (Fig. 2C). The lacunae fuse and are further dilated by the influx of maternal blood. By the 9th week this intervillous space is fully developed (Fig. 2C) (Moore and Persaud 1998).

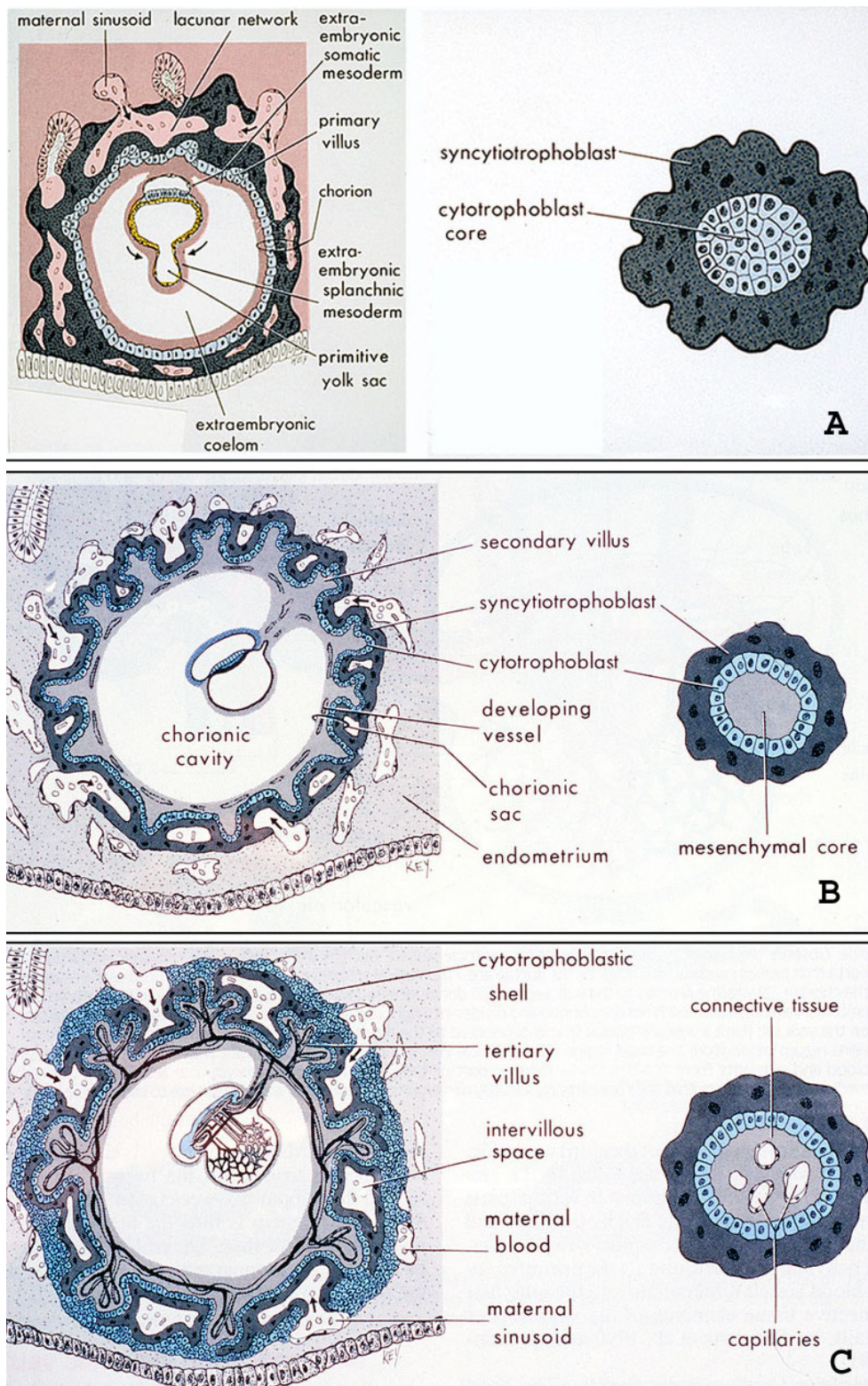


Fig. 2 A, B and C. Formation of the villi. From Moore KL: *The developing human*. Fourth Edition. W.B. Saunders Company; 1988. p. 42, 43 and 62.

3.3 Different cytotrophoblast cell types

All trophoblast cells residing outside the placental villi are called extravillous trophoblast (EVT). There are a number of morphologically and functionally distinct EVT cell types. The importance and distinction of these cell types is still debated. Kaufmann (2003) makes the following distinctions (Fig. 3). At the tip of the villi the EVT forms proliferating clusters of stem cells, so-called cytotrophoblast cell columns. Non-proliferative, invasive daughter cells of the cell columns invade the uterine decidua as interstitial trophoblast. The invasive EVT cells infiltrate the spiral arterial walls in the placental bed and partly replace the endothelial cells, transforming into endovascular trophoblasts. The invading cytotrophoblasts eventually stop migrating and fuse to form polynuclear giant cells in the decidua and the myometrium. Some cytotrophoblasts in the villi do not invade, but end as residual cytotrophoblasts. In vitro cell culture studies indicate that the mononuclear EVT have proliferative and infiltrative properties while the fused giant cells are non-infiltrating and have increased hormone production.

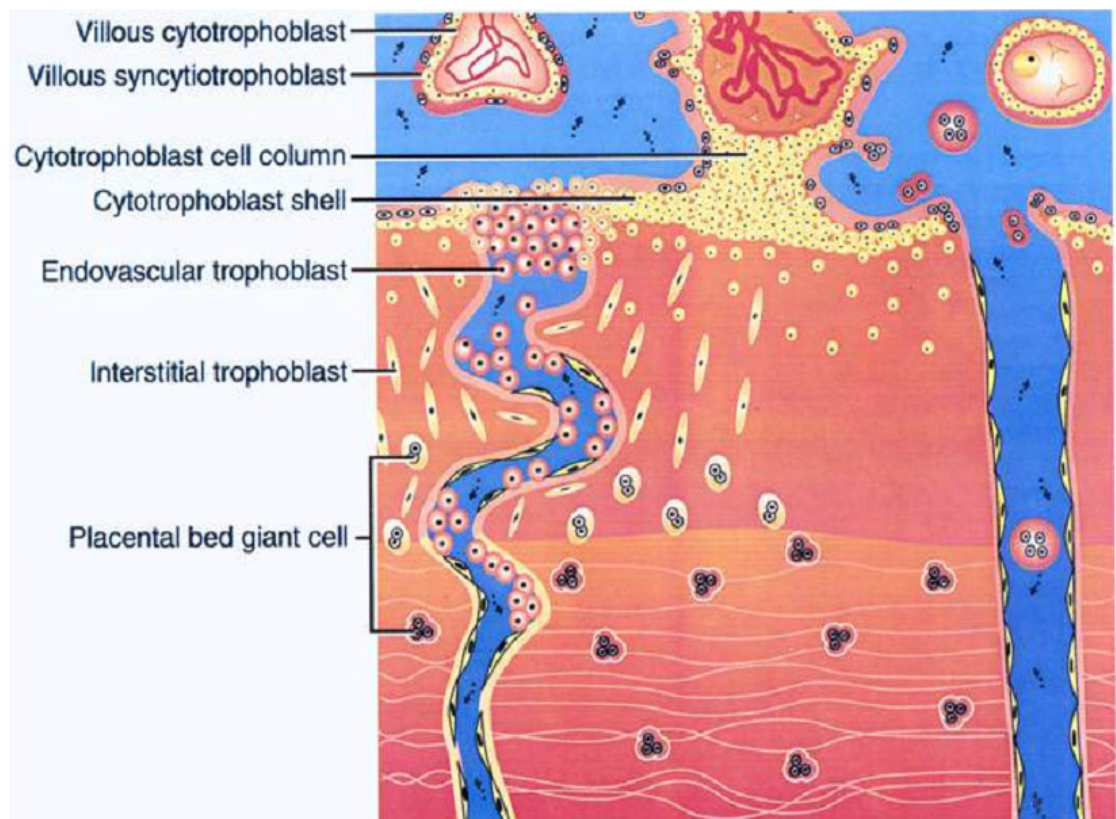


Fig.3. Different trophoblast cell types (From Loke, King 1995)

3.4 Remodelling of maternal spiral arteries

The maternal arteries of the decidua are either spiral arteries or basal arteries. They all branch from the arcuate arteries in the myometrium (Brosens et al 1972). From the site of implantation, the EVT migrate towards the spiral arteries and into the walls of these arteries, partly replacing the endothelial cells. During this process, the spiral artery smooth muscle layer is transformed from a dense muscle coat to a fibrinoid wall with or without evident smooth muscle cells. This process is referred to as remodelling (or physical adaptation), and the elements of this process is still debated. Normally, the remodelled spiral arteries are no longer under maternal vascular control, have an altered morphological appearance. Kaufman now refers to them as uteroplacental vessels. These wide and low resistance vessels allow a tenfold increase of blood flow. In this way efficient blood supply for the fetus is achieved regardless of alterations in the mother's blood flow. This is especially important in the third trimester of the pregnancy; the fetal growth phase.

Complicated pregnancies like miscarriages, intrauterine growth restriction (IUGR)¹ and pre-eclampsia (PE)² are thought to partially relate to trophoblast dysfunction and a defect remodelling of the spiral arteries.

In normal pregnancies the invasion and migration of EVTs and the remodelling of spiral arteries are closely regulated. The invasion is controlled by both intrinsic factors in the trophoblast cell and extrinsic factors in the maternal environment in the decidua. The invasion is a balance between trophoblasts invading as far as possible and the limitations caused by the maternal immune system. (King and Loke 1999). The intrinsic factors are genetic and include adhesion molecules ($\alpha 1\beta 1$ and $\alpha v\beta 3$) and endothelial surface molecules (VE-cadherin, VCAM-1, PECAM-1). In pre-eclampsia the EVTs still express typical stem cell antigens, like $\alpha 6\beta 4$ and E-cadherin (Zhou 1997). EVTs also express HLA-G instead of the ordinary HLA molecules. Maternal uterine NK cells (Eide 2006) in the decidua are shown to express receptors for HLA-G. Binding of HLA-G to these receptors inhibits the NK cells, making the decidua an

¹ intrauterine growth restriction (IUGR) is defined as birth weight below 10th percentile for gestational age. It can be due to fetal chromosomal abnormalities or placental failure due to placental ischemia and infarctions.

² Pre-eclampsia (PE) is a clinical condition defined as maternal blood pressure above 140/95 and proteinuria. It is seen in 2nd and 3rd trimester.

area protected from the maternal immune system. In pre-eclamptic placentas the HLA-G expression is defective (Goldman-Wohl 2000).

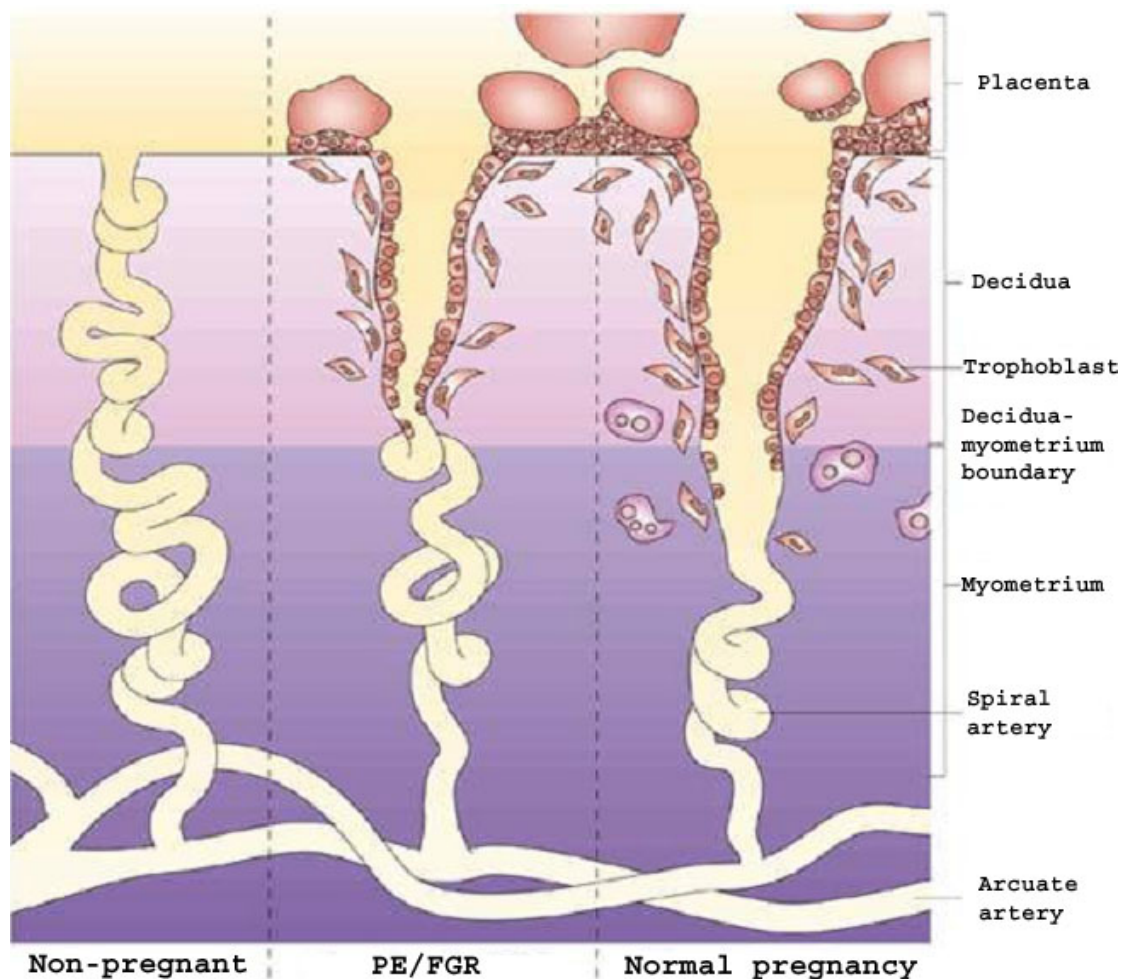
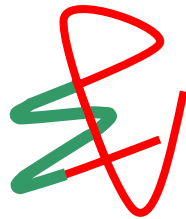


Fig.4 Remodelling of spiral arteries: Brown trophoblasts invade the decidua and migrate towards the spiral arteries, normally transforming them to wide, low resistance vessel-channels. In preeclampsia (PE) and fetal growth restriction (FGR) the trophoblast invasion and remodelling is defect (Nature Medicine)

The genetic makeup of mother and child probably works together through these mechanisms. Some individuals have a genetic susceptibility for PE (Lie et al 1998), showing that genetic makeup is important for the remodelling process. The genes in the process described above are turned on and off by transcription factors both spatially and temporally. By gene inactivation of transcription factors such as Mash2 (Tanaka 1997), GATA2 and GATA3 (Ma 1997) in mice, they are shown to be important for placental development and trophoblast function, as demonstrated in cell culture studies (Loregger 2003).

3.5 Homeobox genes

Homeobox genes are a large family of transcription factors found to control cellular processes such as differentiation, migration, proliferation and invasion in many animal model systems and in humans. The homeobox is a conserved 180bp DNA sequence, encoding the homeodomain part of a protein, which has the ability to bind DNA. The homeobox is highly conserved through evolution (Stein 1996).



Protein, homeodomain in green.



Homeobox gene, homeobox in green

Fig.5. Homeobox gene coding for a protein with homeodomain.

While other transcription factors have but one specific binding site and thus turn on only a few genes, the homeobox genes have short (~10 bp) variable binding sites and thus have the ability to turn on many genes.

The pattern of genes turned on will depend on the internal and external context of the cell, including the activity of other homeobox genes. This makes the homeobox genes important for cell differentiation, proliferation, invasion and migration, and thus important in the regulation of placental and embryonic development. Approximately fifty homeobox genes are identified in humans. Most of these are grouped in four clusters on the DNA, but some are scattered around the chromosomes. Five homeobox genes have been cloned from human placenta at Pregnancy Research Centre (PRC), Royal Women's Hospital, Melbourne: HLX1, DLX4, DLX3, MSX2 and MOX2, all located outside the 4 main clusters. The two first are most highly expressed and therefore the focus of many studies (Quinn 1999). According to Quinn, the protein expression of HLX1 and DLX4 immunohistochemically has been shown in the following cell types:

| Trophoblast cell types in placenta | HLX1 | DLX4 |
|---|-------------|-------------|
| Residual villous cytotrophoblasts | + | + |
| Syncytiotrophoblasts | - | + |

| | | |
|---|---|----|
| Column cytotrophoblasts | + | + |
| Interstitial cytotrophoblasts | + | + |
| Endovascular cytotrophoblasts | + | + |
| Multinuclear cytotrophoblasts (Giant cells) | + | +? |
| Endothelial cells fetal and maternal | + | +? |

3.6 Importance of homeobox genes in placental development

Murthi and colleagues from PRC (2006) did reverse transcriptase PCR, real time PCR, and western blots on normal and IUGR placenta samples. Consistently a drop in HLX1 levels was shown in IUGR compared to normal. Low levels of HLX1 expression in endothelial cells and extravillous cytotrophoblasts make this a candidate gene responsible for the lesser degree of remodelling of spiral arteries in IUGR placenta samples. Elevated levels of DLX4 were seen in syncytiotrophoblasts, areas with extensive apoptosis in IUGR, suggesting that DLX4 plays a role in the process of apoptosis (Personal communication, Padma Murthi). There thus may be an association between alterations in HLX1 and DLX4 expression levels and pathological development of the human placenta.

This does not, however, prove a *causal* relationship between these variables, nor the direction of an eventual causal relationship. By targeted gene inactivation of the Hlx homeobox gene, which is the HLX1 equivalent in mice, the placental structure and vasculature do not develop properly (Murthi 2004). This establishes a causal relationship in mice.

In human trophoblast cell culture, HLX1 can be inactivated by siRNA technique. This makes it possible to investigate which effects a drop in HLX1 levels has on trophoblast functions. A crucial function in trophoblasts is the ability to proliferate. siRNA studies show that when HLX1 is inactivated, there is reduced trophoblast proliferation (Sankaran, Murthi and Kalionis, unpublished data). This could reduce the pool of EVT's available for invasion. Low levels of HLX1 may also influence the trophoblasts ability to migrate and invade. Homeobox genes HLX1 and DLX4 may thus play important roles in placental development and may control important processes in trophoblast invasion and spiral artery remodelling.

3.7 Importance of homeobox genes in placental development

In a spontaneous abortion, the body rejects the conceptus due to chromosomal anomalies, infections and/or placental pathology. Missed abortion represents a condition where the defect conceptus, often without a fetus, is not rejected. We hypothesize that these clinical conditions represent different degrees of trophoblast dysfunction. Therapeutic abortions will have no clinical indication of trophoblast dysfunction, and thus represent normal controls.

We propose that IUGR and PE are conditions caused by the same, but milder, trophoblast dysfunction as in spontaneous and missed abortions, and that this process starts in first trimester. Alteration in the expression of the homeobox genes HLX1 and DLX4 in the EVT's play an important role in IUGR and PE. In term placentas the expression of HLX1 drops in PE and IUGR compared to normal pregnancies, while the DLX4 expression rises (personal communication, Bill Kalionis). Our hypothesis is that alterations of expression of these genes will also be seen in the first trimester in spontaneous and missed abortions.

We expect some of the spontaneous abortions to be caused by defect trophoblast function and therefore express less HLX1 and more DLX4 in the EVT's compared to samples from therapeutic abortions. The therapeutic abortions are considered normal controls. Missed abortions will always be caused by defect placentation (personal communication, Bill Kalionis). These samples will therefore have consistent and high expression of HLX1 but low DLX4 expression.

4. Pilot study

4.1 Aim of study

The aim of this study was to identify remodelled spiral arteries in placental bed material from normal and pathologic first trimester arteries and familiarise with the morphological picture of the remodelling process with respect to trophoblast action. Secondly we wanted to test two PRC-generated rabbit polyclonal antibodies to homeobox genes HLX1 and DLX4. We wanted to explore if the antibodies could be used in a study on expression and action of these homeobox genes in the process of spiral artery remodelling on formalin fixed, paraffin embedded material.

4.2 Patient material

Archive material from the human placental bed and placental tissue from a series of missed abortions, spontaneous abortions and therapeutic abortions in first trimester pregnancies was used in the study. The material was routinely collected by cervical dilatation and curettage of the endometrium and sent to the Department of Pathology, Ullevål University Hospital for diagnostic purposes. The placental bed material from therapeutic abortions was selected as normal controls. The material was made anonymous, identifiable only by the clinical information of gestational data and type of abortion.

4.3 Methods used at Ullevål University hospital

The tissue was fixed in 5% formalin and paraffin embedded. 5µm sections from the paraffin block were routinely stained with hematoxylin and eosin (HE). According to morphological criteria (Eide 2006), we selected and marked areas containing remodelled spiral arteries in these sections. The corresponding areas were punched out of the paraffin blocks and collected in tissue micro arrays (TMAs). 5µm serial sections were cut from the TMAs.

For morphological identification of the remodelled spiral arteries and the various cell populations, TMAs were HE stained. For a more precise identification of the trophoblasts in relation to the remodelling process with loss of smooth muscle medial cells and dilatation of the arteries, a 5µm section from the TMAs were also double immunostained in an automated slide stainer (Ventana Medical System Inc, Tucson, AZ, USA). The first step included primary anti-cytokeratin antibody CK7

(Dako, Copenhagen, Denmark 1/300) and smooth muscle actin (Dako 1/500). The secondary antibodies in both steps were biotinylated (with a streptavidin-biotin complex), and the binding sites were visualized by a 3c3-diaminobenzidine HCl (DAB) chromogen in the first step (iVIEW DAB Detection Kit, Ventana Med System Inc) and alkaline phosphatase in the second step (Ventana Enhanced Alkaline Phosphatase RED Detection Kit, Ventana Medical Systems Inc). All sections were counterstained in haematoxylin. The HE staining and a double immunostaining with CK7/smooth muscle actin was performed at the department of pathology at Ullevål University Hospital prior to us going to PRC at Royal Women Hospital, the University of Melbourne. To PRC we brought with us these stained TMA-sections in addition to unstained serial TMA sections for homeobox protein immunostaining by us in Melbourne.

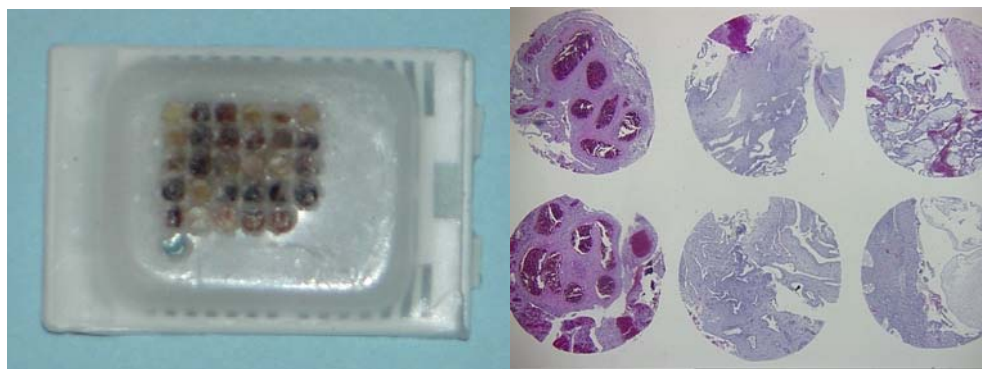


Fig. 6 Tissue micro array (TMA) with punched out areas from the placental bed

4.4 Immunohistochemical protocol at Pregnancy Research Centre

The method we used for the immunohistochemical stainings at PRC/RWH under supervision of research fellow Padma Murthi was adopted from L. M. Quinn (1998). The sections were dewaxed by heating for 30 minutes at 37°C, and then placed in xylene for 30 minutes and then fresh xylene for 10 minutes. Then we rehydrated the sections by going through 100%, 90%, 70% and 50% ethanol, and finally in de-ionised distilled water; each step 2 times for 3 minutes. Following TBS wash, we left the sections for proteinase K digestion for 15 minutes at 37°C, and blocked them immediately in 10% skim milk for 1 hour. The sections were incubated with the primary antibodies overnight at 4°C.

The following day we washed the sections in TBS and then the primary antibody was added by incubation for 30 minutes with biotinylated polyclonal anti-

rabbit antibody. After TBS wash, the reaction was amplified using the TSA amplification kit following the manufacturer's instructions. The colour reaction was prepared using streptavidin-alkaline phosphatase for 30 minutes, before overnight incubation in NBT/BCIP at 4°C. Finally we counterstained the sections with methyl-green for 5 minutes and mounted the slides with 80% glycerol.

5. Results

After some training, we managed to identify the remodelled spiral arteries in the placental bed material.

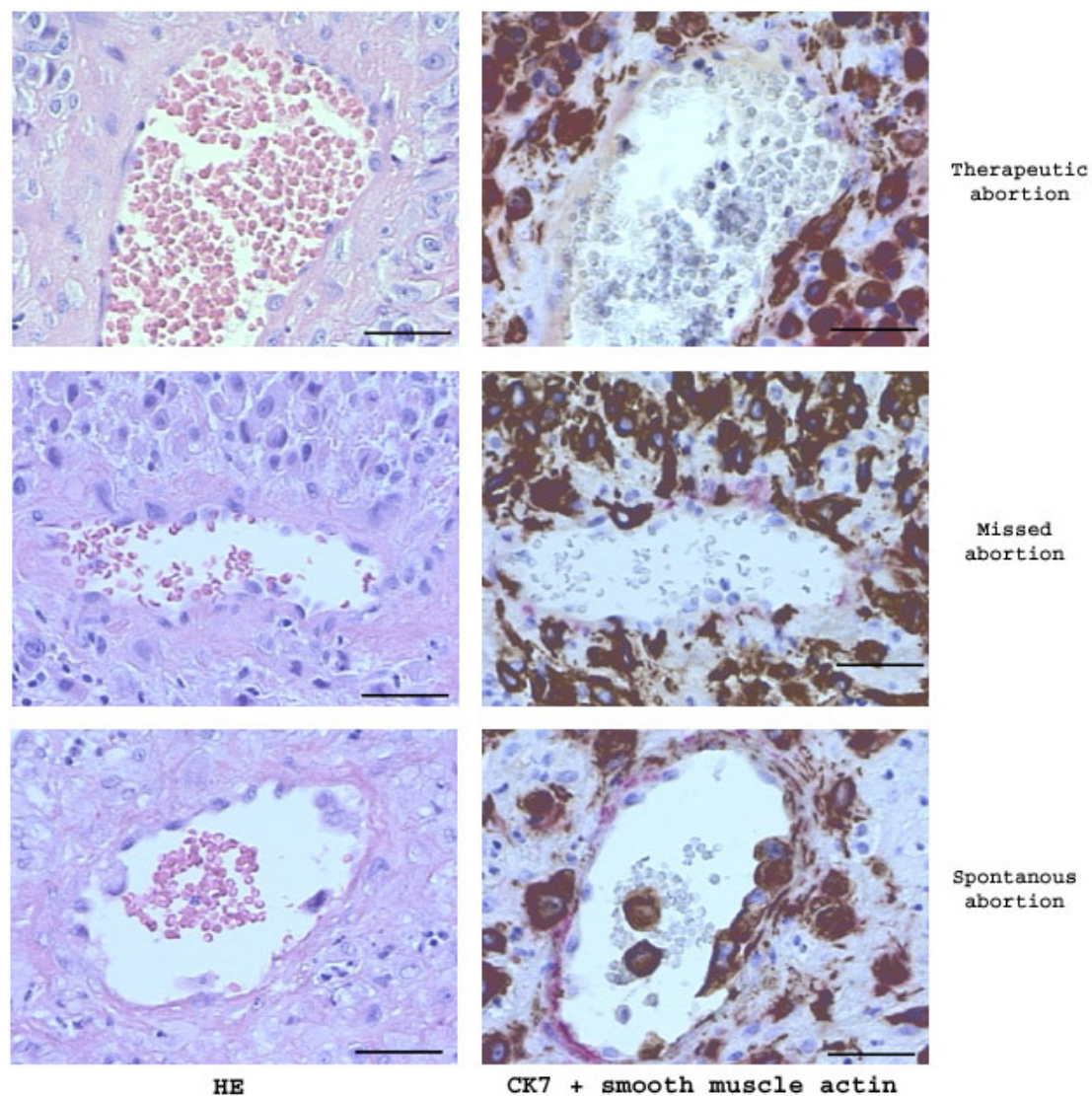


Fig.7 TMA sections containing remodelled spiral arteries from the placental bed in normal and pathological first trimester pregnancies. HE staining (left) and double immunostained for smooth muscle actin (red) and cytokeratin CK7 (trophoblasts). Magnification X 200.

Remodelled spiral artery

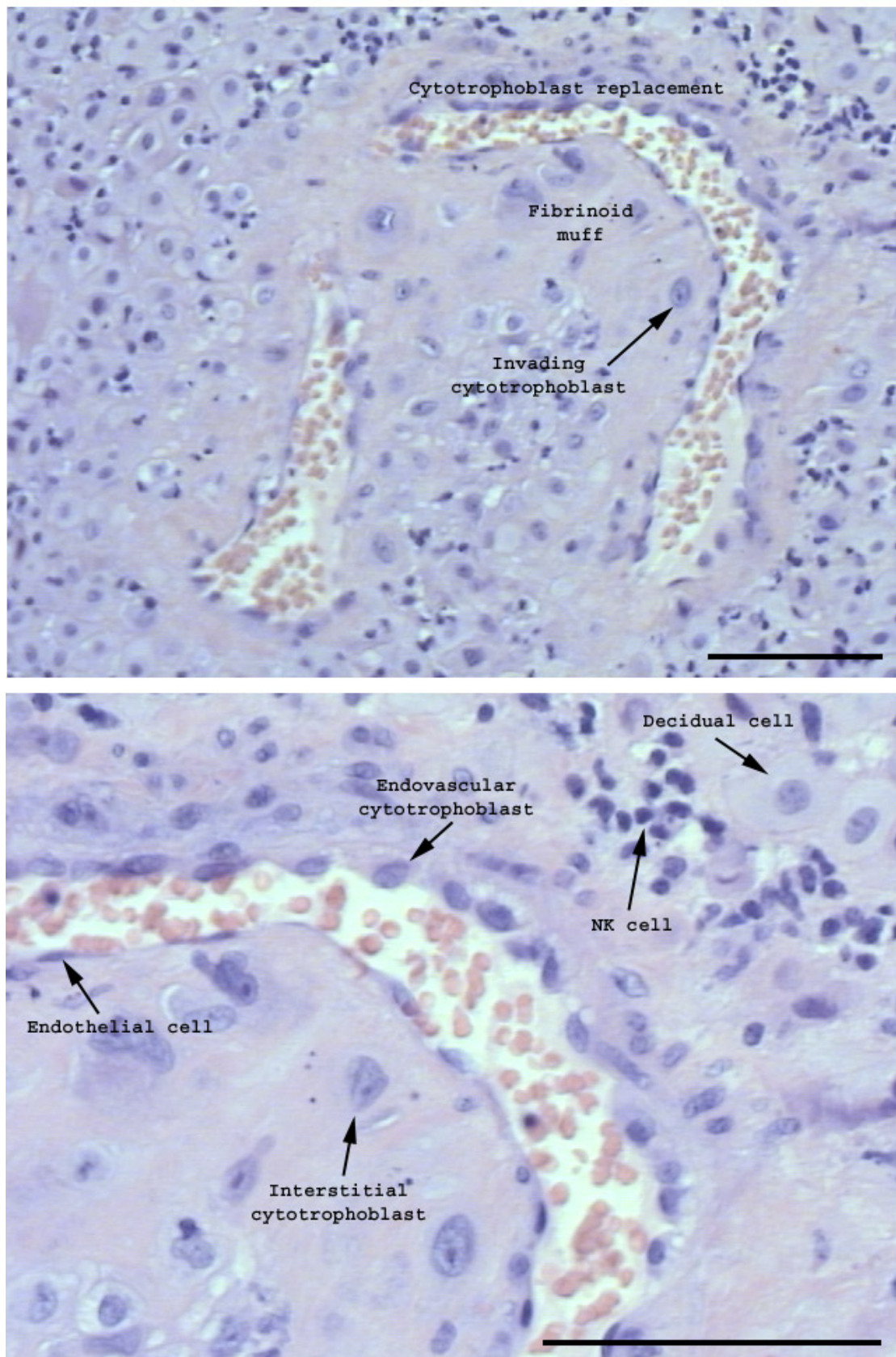


Fig.8 Identification of the important cells in the remodelling process, HE section from TMA. X 200.

We had, however, major problems in making the immunostaining with the antibodies against HLX-1 work in a stable way. We did not manage to visualize the immunostain with the DLX4 antibody. All the steps in the procedure were repeatedly tested, partly with appropriate modifications. The results were unfortunately the same, and we concluded that our problems related to the quality of the antibodies and that they were not suitable for immunohistochemical studies on formalin fixed material with the techniques used at PRC.

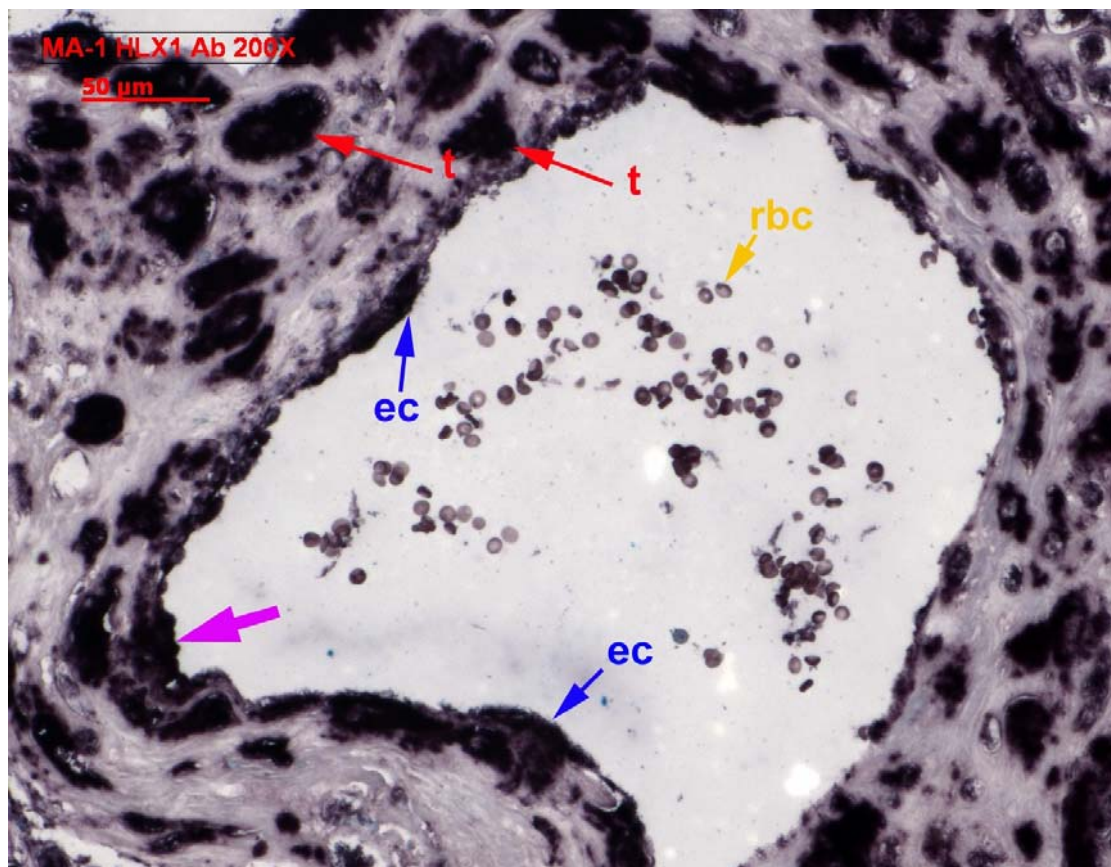


Fig 9. A high magnification view of a partially transformed spiral artery is shown. Trophoblast cells (t) are shown penetrating the smooth muscle layer. Red blood cells (rbc) in artery lumen are shown. Endothelial cells (ec) are known to stain with HLX1. The characteristic stretched endothelial cells are indicated. When trophoblast cells fully transform the artery, these cells are replaced by larger, rounder trophoblast endovascular cells that are exposed to the lumen (purple arrow).

6. Conclusion and discussion

Morphologically, after some training, we relatively easily managed to identify the remodelled spiral arteries in the routine stained placental bed material. The important cells in the remodelling process (endothelial cells, invading/interstitial extravillous cytotrophoblasts, decidual cells and NK cells) were seen in the HE stained sections (see Fig. 8). By immunostaining with cytoskeleton antibody CK7 a much larger number of Trophoblasts were seen and the identification was easier and definitive. The identification of smooth muscle cells in the remodelled vessel walls also was greatly eased (see Fig. 7).

The tedious and not very successful laboratory work with the homeobox antibodies taught us much about the pitfalls in the work with protein markers on formalin fixed, paraffin embedded material in immunohistochemical studies. In lack of immunoreactions in the placental bed with the homeobox antibodies, we repeatedly tested all the various steps in process. The final conclusion was that the problem was related to the antibodies.

On formalin fixed material the epitopes are vulnerable. This can thus explain our difficulties. In immunohistochemical studies, the gold standard is fresh frozen or ethanol/acetone fixed material, which are thought to leave the epitopes (antigen determinates) in a native state. All sorts of fixation procedures can mask the epitopes. Much of the technical challenges in immunohistochemical studies on formalin fixed material lies in the demasking of epitopes. Various chemical procedures and microwaving of the sections are constructed for that means. In our pilot study, we quality tested all the various steps in the Quinn method used at PRC. Due to short time allocated to the Australian part of the study, we did not test out other antigen retrieval methods. We did, however, learn a lot about laboratory work and the vulnerability of the methods.

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